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Isolation and characterization of the insecticidal constituent acorone from *Acorus calamus* (Araceae)

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Abstract: The stored-grain insect pests can cause serious quantitative and qualitative losses of stored-grain. Because of the negative effects of the synthetic insecticides, there is an urgent need to develop new types of environmental-friendly insecticides, of which plant has become a new potential resource. Our previous study showed that the extract of the traditional medicinal plant *Acorus calamus* had toxicity to stored-grain insects and (*Z*)-asarone was found to be one of the active constituents from its extract. In this study, the extract of *A. calamus* was separated and another active constituent of *A. calamus* extract was obtained by choromatographing and bioassay-guiding its contact toxicity to *Sitophilus zeamais*. Using spectroscopic analysis, including GC-MS, IR, ^{1}H NMR, ^{13}C NMR, and single crystal X-ray diffraction, the active constituent was identified as a single sesquiterpenoid compound acorone (1- isopropyl- 4, 8-dimethyl- spiro ; 4. 5] decane - 2, 7 - dione). When *S. zeamais* was treated for 96 h with acorone at the concentration of 314.54, 251.63, 188.72, 125.82 and 62.91 μg/cm^2, its mortalities were 85.6%, 72.2%, 53.3%, 38.9% and 15.6%, respectively. LD_{50} at 96 h after treatment was 158.00 μg/cm^2. We so concluded that acorone has significant contact toxicity to *S. zeamais* and is another active constituent besides (*Z*)-asarone.

Key words: *Acorus calamus*; acorone; active constituent; contact toxicity; *Sitophilus zeamais*

1 INTRODUCTION

Botanical insecticides have been used to control pests for centuries (Hao and Ge, 1999; Thacker, 2002). During the 20th century, the botanical insecticides were progressively replaced by synthetic insecticides owing to the efficacy, speed of action, ease of use and low cost of the synthetic insecticides. However, long-term use of synthetic insecticides has caused environmental contamination, toxicity to non-target organisms and resurgence of target pests (Marco *et al.*, 1987; Perry *et al.*, 1998; National Research Council, 2000; Isman, 2006). All these problems make scientists turn back to search new environmentally friendly botanical insecticides. Plant has become an important potential resource to develop new environmentally friendly pesticides.

Over one thousand species of insect pests infesting stored-products are found in the world, and these pests cause serious quantitative and qualitative losses of stored-products (Rajendran, 2002). Plants and their extracts have been used for protecting stored products from insect pests (Adler *et al.*, 2000; Weaver and Subramanyam, 2000; Isman, 2006).

To find the main active constituents of the plant extract, and take them as the pilot chemical compounds to develop the new bionic pesticides were one of the main uses of the botanical insecticides (Belmain *et al.*, 2001). *Azadirachta indica* A. Juss was a very successful example. Azadirachtin was isolated from *A. indica* and showed great activity to the pests (Benge, 1986). Eight kinds of constituents were isolated from *Rhododendron molle*, and rhodojaponin-Ⅲ had the most significant insecticidal activity (Liu and Pan, 1989; Klocke *et al.*, 1991). Celangulin I - V were identified from *Celastrus angulatus*, and they showed repellent, contact or narcotic activity against the pests (Wu *et al.*, 1993; Wu and Li, 1994). Take nicotine, isolated from *Nicotiana tobacum*, as the pilot chemical compound, the new insecticides had been found (Yang *et al.*, 2008).

Acorus calamus L. (Family Araceae), commonly known as sweet flag, is a perennial shrub

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growing in damp marsh. *A. calamus* is mainly used as a medicinal plant, and its roots and rhizomes have been used in Indian and Chinese traditional medicine for years. It can modulate immune system and has thus been used for treatment of insomnia, melancholia, neurosis, epilepsy, hysteria and loss of memory; it also has anti-aging effect on senescence (Agarwal *et al.*, 1956; Nishiyama *et al.*, 1994a, 1994b; Zhang *et al.*, 1994; Mehrotra *et al.*, 2003).

Like other medicine plants, extracts from *A. calamus* have antibiotic effects against bacteria and insect pests. The crude extracts of *A. calamus* have high activity against multidrug-resistant enteric bacteria (Ahmad and Aqil, 2007). The extracts from *A. calamus* present high contact and fumigation activity against agricultural pests, including main stored-product pests such as *Lasioderma serricorne*, *Callosobruchus phaseoli*, *Sitophilus oryzae*, *Prostephanus truncatus*, *Sitophilus granarius* and *Callosobruchus chinensis* (Schmidt and Streloke, 1994; Paneru *et al.*, 1997; Rahman and Schmidt, 1999; Kim *et al.*, 2003a, 2003b; Yao *et al.*, 2008).

Our previous research has shown that the ethanol extracts of *A. calamus* have strong repellency and contact toxicity to *Sitophilus zeamais*, and the petroleum ether fraction of the ethanol extract was found to be the most effective fraction, and one of the active constituents is characterized as (*Z*)-asarone (Yao *et al.*, 2008). In this study, the petroleum ether fraction of *A. calamus* was separated with the intention to identify more possible active constituents and provide more information for the exploitation of *A. calamus*.

2 MATERIALS AND METHODS

2.1 Insects

The experimental *Sitophilus zeamais* Motschulsky beetles were collected from Wuhan Branch, Central Grain Reserves, and raised in the laboratory for more than one year without exposure to any insecticide. The *S. zeamais* were reared on wheat at 27 ± 1℃, RH 75% ± 5%, and a photoperiod of 16L:8D. For the bioassay, 3-week-old beetles without prior starvation were used.

2.2 Isolation and identification

Our previous research had shown that petroleum ether fraction of *A. calamus* was the active fraction (Yao *et al.*, 2008).

Using the method described by Yao *et al.* (2008), the petroleum ether fraction (150 g) was got, and was chromatographed on a silica gel column [100 – 200 mesh, 1 300 g, 7.5 cm i. d. (internal diameter) × 110 cm], and successively eluted with a stepwise gradient of petroleum ether: ethyl acetate (100:0, 99:1, 95:5, 90:10, 85:15, 80:20, 70:30, 60:40, 10:50 and 0:100, v/v). The eluent was analyzed using thin layer chromatography (TLC) (silica gel G), and fractions with a similar TLC pattern were pooled. The bioactive fraction (7 g) was re-chromatographed on a silica gel column (200 – 300 mesh, 210 g, 6 cm i. d. × 83 cm), using petroleum ether: acetone (25:1, 15:1, 5:1 v/v), and 5.61 g bioactive fraction was obtained. After that the bioactive sub-fraction (5.00 g) was further re-chromatographed on a silica gel column (200 – 300 mesh, 200 g, 2 cm i. d. × 80 cm), using petroleum ether : acetone (25:1)(v/v). Finally, a potent active constituent of 4.68 g was isolated.

The structure of the active constituent was determined using spectroscopic analysis and single crystal X-ray diffraction. The ^{1}H NMR and ^{13}C NMR spectra were recorded with a MERCURY PLUS-400 spectrometer at 400 MHz and 100 MHz, respectively. IR spectra were obtained on a 670FT-IR spectrometer and GC-MS spectra on a VARIAN CP-3800 and SATURN-2200MS/MS spectrometer. Single crystal X-ray diffraction was recorded with BRUKER SMART APEX-CCD.

2.3 Contact toxicity

The extracts were mixed with acetone to make up different concentrations of solution for contact activity test. For each toxicity assay experiment, 1 mL of acetone solution was spread evenly onto filter papers (9 cm diameter) in a fume hood. After dried in the fume hood for 2 min, each filter paper was placed in a Petric dish (9 cm diameter), in which 30 *S. zeamais* adults were also placed. Each Petri dish was covered with a glass cup which was smeared with polytetraflu-oroethylene in order to prevent the test insects from escaping. A dry filter paper loaded with 1 mL of acetone was used as the control. All treatments were repeated six times. The setup was maintained under the conditions as described above. Cumulative mortality was recorded at 24 h, 48 h, 72 h and 96 h after treatment. Insects were presumed dead if they did not move when touched with a brush. Percentage insect mortality was corrected using the Abbott (1925) formula.

2.4 Data statistics and analysis

The percentage mortality was arcsine transformed before analyzed by PROC NPAR1WAY Wilcoxon of the SAS statistical package. The mean of percentage mortality was compared using the non-parametric Nemeny test (SAS Institute, 1990). Means (±*SE*) of untransformed data were reported.

Probit analysis using the Maximum Likelihood Programme software was employed in analyzing the dosage mortality response to get LD_{50} (Finney, 1971).

3 RESULTS

3.1 Contact toxicity of different fractions of *A. calamus* extract to *S. zeamais*

The petroleum ether fraction was choromatographed to obtain 10 fractions. The contact toxicity of the 10 fractions to *S. zeamais* was laboratory-assayed, and significant differences were observed at the concentration of 157.20 μg/cm^2 (Table 1). Fraction D caused 100.0% mortality in adult *S. zeamais* after 96 h treatment, while fraction E and B caused 77.8% and 51.1% mortality. The mortalities caused by other fractions were all less than 10%.

Table 1 Contact activity of primary fractions from *Acorus calamus* against adult *Sitophilus zeamais*

Fraction	Mortality (%) (mean ± *SE*)			
	24 h	48 h	72 h	96 h
A	0.0 ± 0.0 c	1.1 ± 1.1 b	3.3 ± 1.9 cd	8.9 ± 1.0 cd
B	1.1 ± 1.1 b	7.6 ± 1.0 a	13.1 ± 2.0 ab	51.1 ± 0.5 bc
C	0.0 ± 0.0 c	1.1 ± 1.1 b	4.4 ± 1.2 c	7.6 ± 1.2 def
D	62.2 ± 2.9 a	96.7 ± 1.9 a	100.0 ± 0.0 a	100.0 ± 0.0 a
E	10.0 ± 1.9 a	18.9 ± 2.9 a	61.1 ± 2.9 a	77.8 ± 2.2 ab
F	0.0 ± 0.0 c	1.1 ± 1.1 b	3.3 ± 1.9 c	5.6 ± 1.1 ef
G	0.0 ± 0.0 c	1.1 ± 1.1 b	2.2 ± 1.1 cd	2.2 ± 1.1 gh
H	0.0 ± 0.0 c	1.1 ± 1.1 b	1.1 ± 1.1 cd	7.8 ± 1.1 de
I	0.0 ± 0.0 c	2.2 ± 1.1 b	3.3 ± 0.0 bc	4.4 ± 1.1 fg
J	0.0 ± 0.0 c	0.0 ± 0.0 b	0.0 ± 0.0 d	0.0 ± 0.0 h

The dose in the experiment was 157.20 μg/cm^2. Each datum represents the mean of six replicates, each setup with 30 adults (n = 180). Means within a column followed by different letters are significantly different at P = 0.05 (Nemeny test). χ^2(24 h) = 26.653, P = 0.002; χ^2(48 h) = 22.493, P = 0.007; χ^2(72 h) = 22.772, P = 0.007; χ^2(96 h) = 26.768, P = 0.002 (PROC NPAR1WAY Wilcoxon).

GC-MS analysis indicated that the main active constituent in fraction D was (*Z*)-asarone, which had been reported by Yao *et al.* (2008); therefore, fraction E was separated in this study in order to identify its active constituent.

Fraction E was re-chromatographed to obtain four sub-fractions E1, E2, E3 and E4. Each sub-fraction showed different contact toxicities against *S. zeamais* under the concentration of 157.20 μg/cm^2 (Table 2). After 96 h treatment, sub-fraction E2 caused 50.1% mortality, which was significant higher than those caused by other sub-fractions ($P \leqslant 0.05$).

Table 2 Contact activity of sub-fractions of E from *Acorus calamus* against adult *Sitophilus zeamais*

Sub-fraction	Mortality (%) (mean ± *SE*)			
	24 h	48 h	72 h	96 h
E1	0.0 ± 0.0 b	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
E2	18.9 ± 2.9 a	21.1 ± 2.9 a	43.0 ± 1.9 a	50.1 ± 2.9 a
E3	0.0 ± 0.0 b	2.2 ± 1.1 b	4.4 ± 1.1 b	5.6 ± 1.1 b
E4	0.0 ± 0.0 b	0.0 ± 0.0 c	1.1 ± 1.1 c	5.6 ± 2.2 b

The dose in the experiment was 157.20 μg/cm^2. Each datum represents the mean of six replicates, each set up with 30 adults (n = 180). Means within a column followed by different letters are significantly different at P = 0.05 (Nemeny test). χ^2(24 h) = 10.735, P = 0.013; χ^2(48 h) = 9.511, P = 0.023; χ^2(72 h) = 9.713, P = 0.021; χ^2(96 h) = 9.663, P = 0.022 (PROC NPAR1WAY Wilcoxon).

The activity of sub-fraction E2 was the highest among all the sub-fractions, therefore it could be concluded that the active constituent existed in sub-fraction E2. Sub-fraction E2 was then further re-chromatographed to obtain an active constituent.

3.2 Identification

The above obtained active constituent was identified by spectroscopic analysis, including GC-MS, IR, ^{1}H NMR and ^{13}C NMR (Figs. 1 – 5).

The characteristics of the constituent were obtained from the spectra information. Molecular formula: $C_{15}H_{24}O_2$. White crystals. EI-MS *m/z* 236 $[M+H]^+$; IR (KBr): 1 736 (C = O), 1 699 (C = O), 1 470, 1 074, 657 cm^{-1}. ^{1}H NMR (CH_3COCH_3, 400 MHz) δ (ppm): 2.48 – 1.47 (m, 12H, 4 × CH and 4 × CH_2), 1.00 (d, J = 6.8 Hz, 3H, CH_3), 0.94 (d, J = 6.4 Hz, 3H, CH_3), 0.88 (d, J = 6.4 Hz, 3H, CH_3), 0.72 (d, J = 6.8 Hz, 3H, CH_3). ^{13}C NMR (CH_3COCH_3, 100 MHz) δ (ppm): 216.9, 211.9, 65.9, 50.2, 45.9, 44.2, 42.1, 40.5, 37.9, 28.3, 25.3, 15.3, 14.6.

In order to further confirm the structure of the compound, its single crystal was cultivated to obtain its crytal structure (Fig. 6).

From the structure analysis, it could be concluded that the compound was a known sesquiterpenoid named as acorone (1-isopropyl-4, 8-dimethyl-spiro; 4. 5] decane-2, 7-dione) (Fig. 7).

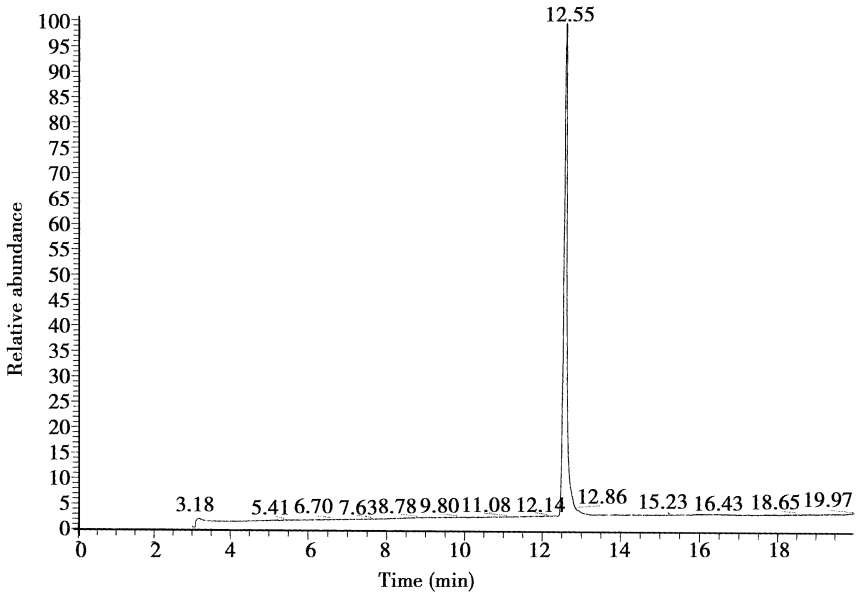

Fig. 1 GC spectra of an active constituent from *Acorus calamus*
GC spectra indicated that the constituent is a single compound.

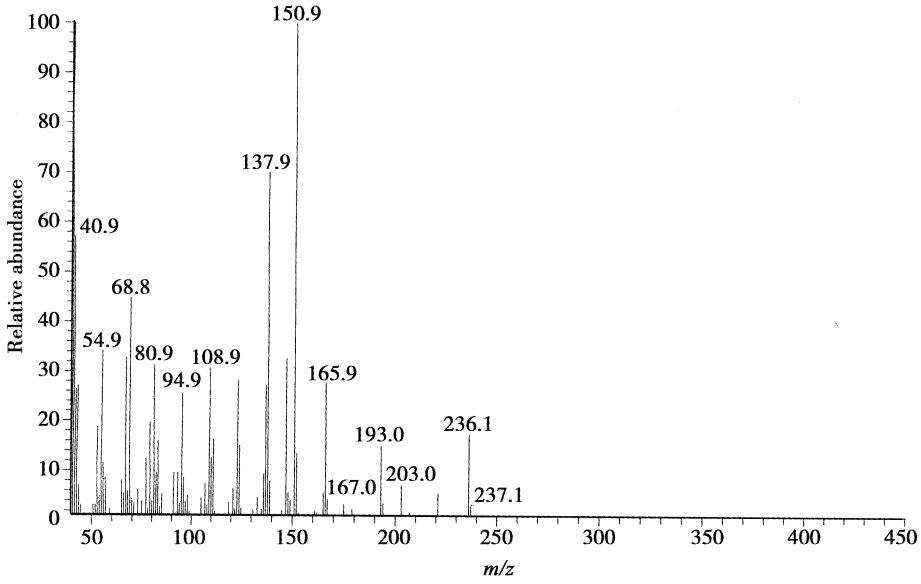

Fig. 2 MS spectra of an active constituent from *Acorus calamus*
MS spectra indicated that the molecular weight of the compound was 236.

3.3 Verification experiment for the contact toxicity of acorone against *S. zeamais*

Contact toxicity of the tested compound on *S. zeamais* were assessed (Table 3). The insecticidal activity was dependent upon the concentration and the exposure time. The compound only caused 28.1% mortality in *S. zeamais* at the highest concentration (314.54 μg/cm^2) at 24 h after treatment, however, caused 60.0% mortality at the same concentration at 72 h after after treatment. At 96 h after treatment, the mortalities at the concentration of 314.54, 251.63, 188.72, 125.82 and 62.91 μg/cm^2 were recorded as 85.6%, 72.2%, 53.3%, 38.9% and 15.6%, respectively. There was significant difference ($P \leqslant 0.05$) in the mortalities among different concentrations at 96 h after treatment. The LD_{50} at 96 h was calculated as 158.00 μg/cm^2.

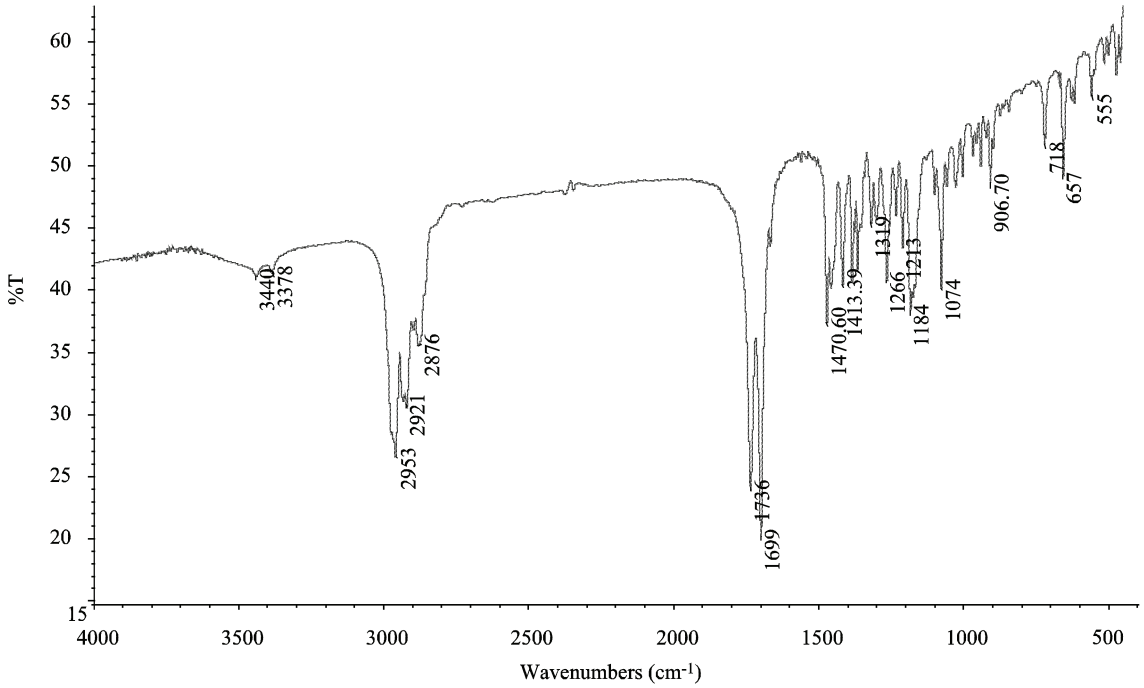

Fig. 3　IR spectra of an active constituent from *Acorus calamus*

IR spectra indicated that the compound contained carbonyl ($>C=O$) and methyl (CH_3-).

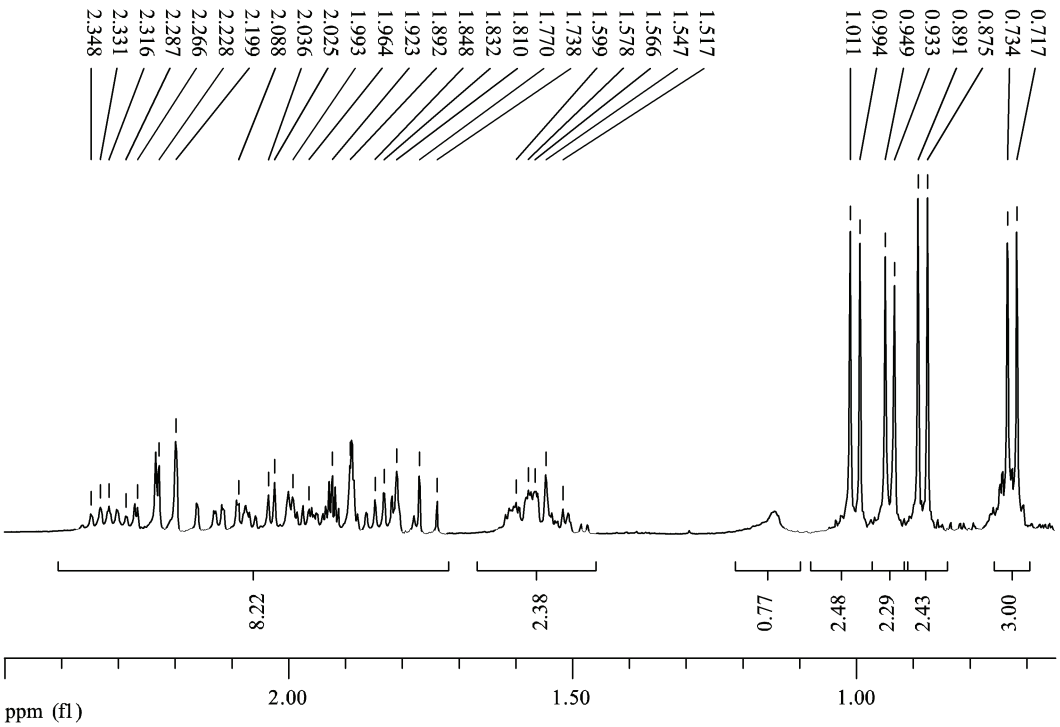

Fig. 4　^{1}H NMR spectra of an active constituent from *Acorus calamus* (CH_3COCH_3)

^{1}H NMR spectra indicated that the compound contained four methyl (CH_3-).

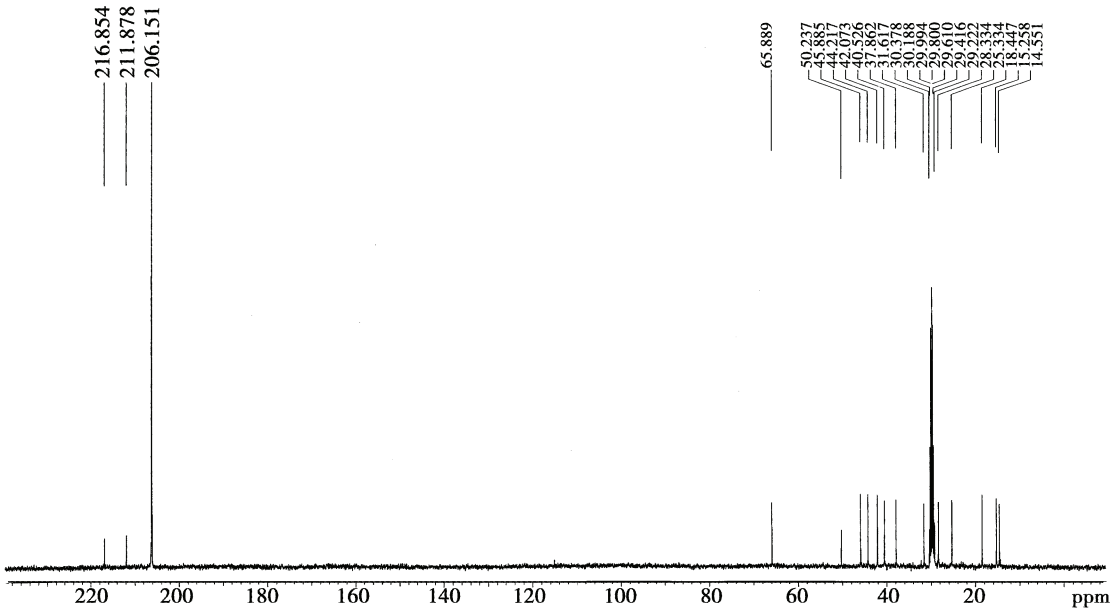

Fig. 5 ^{13}C NMR spectra of an active constituent from *Acorus calamus* (CH_3COCH_3)
^{13}C NMR spectra indicated that the compound contained two carbonyl (>C=O).

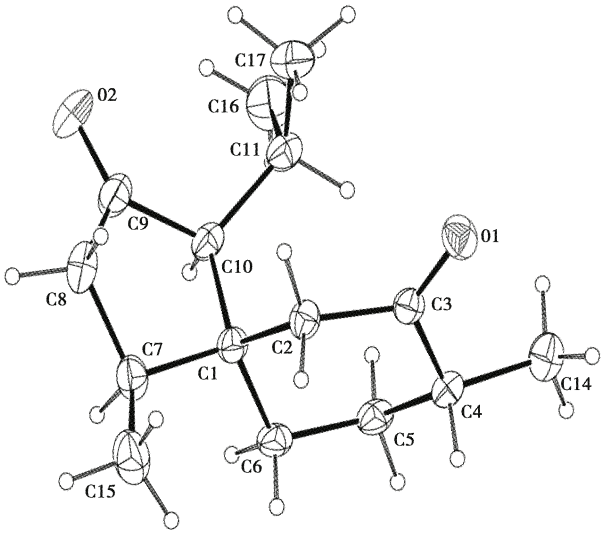

Fig. 6 Crystal structure of an active constituent from *Acorus calamus*

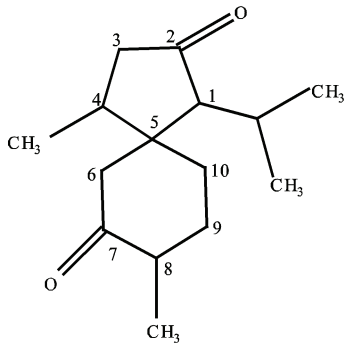

Fig. 7 Structure of 1- isopropyl- 4, 8 -dimethyl-spiro ; 4. 5] decane- 2, 7 - dione

Table 3 Contact activity of acorone against adult *Sitophilus zeamais*

Dose ($\mu g/cm^2$)	Mortality (%) (mean ± *SE*)			
	24 h	48 h	72 h	96 h
314.54	28.1 ± 0.7 a	41.1 ± 2.9 a	60.0 ± 1.9 a	85.6 ± 4.0 a
251.63	12.0 ± 1.5 b	17.8 ± 2.9 b	44.4 ± 4.0 b	72.2 ± 2.9 b
188.72	3.5 ± 3.5 c	7.8 ± 1.1 c	28.9 ± 2.9 c	53.3 ± 1.9c
125.82	0.0 ± 0.0 c	4.4 ± 1.1 d	17.8 ± 1.1 d	38.9 ± 2.9 d
62.91	0.0 ± 0.0 c	0.0 ± 0.0 e	6.7 ± 1.9 e	15.6 ± 1.1 e

Each datum represents the mean (± *SE*) of six replicates, each setup with 30 adults (n = 180). Means within a column followed by different letters are significantly different at P = 0.05 (Nemeny test). χ^2 (24 h) = 12.553, P = 0.014; χ^2 (48 h) = 13.450, P = 0.009; χ^2 (72 h) = 13.524, P = 0.009; χ^2 (96 h) = 13.524, P = 0.009 (PROC NPAR1WAY Wilcoxon).

4 DISCUSSION

Previous studies have shown that the extract of *A. calamus* can be used to control several species of stored-grain insects (Paneru *et al.*, 1997; Rahman and Schmidt, 1999; Kim *et al.*, 2003a, 2003b; Tewary *et al.*, 2005). In this study, we found that the ethanol extract of *A. calamus* has strong repellency and contact activities to *S. zeamais*. It was also found that there is more than one active constituent in the rhizome of *A. zeamais*, mainly distributed over the petroleum ether fraction. So far, two active constituents, (*Z*)-asarone and acorone, have been identified in the rhizome extract of *A. zeamais* in our studies.

Some compounds, such as α and β asarone (Chopra *et al.*, 1965; Mazza, 1985a, 1985b;

Schmidt and Streloke, 1994; Oprean *et al.*, 1998; Yao *et al.*, 2008), sesquiterpenoids (Yamamura *et al.*, 1971; Rohr *et al.*, 1979; Nawamaki and Kuroyanagi, 1996), lectins (Bains *et al.*, 2005), and phenyl indane (Saxena, 1986), have been isolated or identified from extracts of *A. calamus*. Among them, β asarone exhibits pesticidal activities against stored-product pests (Schmidt and Streloke, 1994; Yao *et al.*, 2008).

Acorone is a known sesquiterpenoid and colorless crystal at room temperature. It can be dissolved in most organic solvents and easily dissolved in acetone and petroleum ether. Acorone has been separated and isolated from extracts of *A. calamus* (Sorm and Herout, 1948), and its inhibitory effect on germination of lettuce seeds has also been reported (Nawamaki and Kuroyanagi, 1996). The synthetic technology for acorone has been studied (McCrae and Dolby, 1977; Martin and Chou, 1978). However, the pesticidal activity of acorone has not been reported up to date. In this study, acorone is separated and identified from extracts of *A. calamus* with bioassay guiding, and its pesticidal activity is first reported.

In this study, fraction E of the concentration 157.20 μg/cm^2 caused 77.8% mortality in adult *S. zeamais* at 96 h after treatment, while the most effective sub-fraction E2 of fraction E only caused 50.1% mortality. These results indicate that there are various constituents, including the main active constituents and co-operative active constituents, in the rhizome of *A. calamus*. These active constituents present unequal effects on insects, and the interactions among the constituents are very complex. The LD_{50} of acorone against *S. zeamais* is 158.00 μg/cm^2 at 96 h after treatment, compared to the 28.03 μg/cm^2 of (*Z*)-asarone against *S. zeamais*. Therefore, it can be concluded that (*Z*)-asarone is the main active constituent in *A. calamus*. Whether (*Z*)-asarone has cooperative action with acorone needs to be further studied.

A. calamus is a perennial herb with large biomass, and widely distributed in China. This would provide a rich source for the exploitation of *A. calamus*. Further research on the insecticidal activities of *A. calamus* to other stored-grain insects, the modes of action and the safety issues need to be done.

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水菖蒲杀虫活性组分菖蒲螺酮的分离与鉴定

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摘要: 储粮害虫的危害造成储粮严重损失, 化学农药的种种弊端使开发新型环境友好型药剂非常迫切, 植物由于其自身的特点, 成为开发新型药剂的重要来源。我们的前期研究表明, 水菖蒲 *Acorus calamus* 提取物对多种储粮害虫具有明显的触杀活性, 并且分离得到了其主要活性组分 β-细辛醚。本研究采用硅胶柱层析法, 并以对玉米象 *Sitophilus zeamais* 的触杀活性进行追踪, 对水菖蒲提取物进行分离, 得到另一活性组分。经气相色谱-质谱联用、红外吸收光谱和核磁共振波谱方法鉴定, 该活性组分为单体化合物菖蒲螺酮(1-异丙基-4,8-二甲基螺[4.5]癸-2,7-二酮)。将菖蒲螺酮以 314.54, 251.63, 188.72, 125.82 和 62.91 μg/cm^2 的浓度处理玉米象 96 h 后, 玉米象的死亡率分别为 85.6%, 72.2%, 53.3%, 38.9% 和 15.6%。处理 96 h 后, 菖蒲螺酮对玉米象成虫的 LD_{50} 为 158.00 μg/cm^2。这些结果说明菖蒲螺酮对玉米象具有明显的生物活性, 是水菖蒲提取物中除 β-细辛醚以外的又一活性组分。

关键词: 水菖蒲; 菖蒲螺酮; 活性组分; 触杀活性; 玉米象

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